MEETING THREAT DETECTION NEEDS FOR THE ARMY: USING CONJUGATED POLYMERS TO IMPROVE DIAGNOSTICS

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ABSTRACT

The focus of this work is on the development of an integrated biological detection system that can meet the needs of Army to detect biological threats. This detection system will be based on a heterogeneous microarray format, which allows for the simultaneous detection of multiple threat agents, and cationic conjugated polymers (CCPs), which serve either to enhance microarray systems (both commercially available and custom-printed) by increasing signal output of a reporter dye via Förster resonance energy transfer (FRET) or to provide a means for reporting in label-free microarray systems that could greatly reduce the cost for time and materials. For labeled systems, amplified signal intensities of reporting dyes should translate to either a higher sensitivity or lower requirements for optical specifications. Simplification of the optics would not only reduce the overall cost of testing, but opens the door for the overriding goal of design and development of portable and/or point-of-care testing devices. These types of diagnostic devices would have the potential to perform field-deployable, simple, and cost-effective testing in a timely fashion for bio-threat agents.

1. INTRODUCTION

Methods for the real-time, specific detection of DNA are of considerable interest (Wang, 2000; Umek *et al.*, 2001; Schork *et al.*, 2000). Homogeneous real-time detection of DNA can be achieved through real-time PCR using hybridization probes such as molecular beacons (Tyagi and Kramer, 1996), Taqman (Holland *et al.*, 1991), and Scorpion (Thelwell *et al.*, 2000). The advent of heterogeneous hybridization probe platforms such as DNA microarrays allow for analysis of changes in genome-wide patterns of gene expression. Those cDNA microarrays are often used to compare the gene expression profiles of up to four samples hybridized to a single chip (Service, 1998; Niemeyer and Blohm, 1999; Alizadeh *et al.*, 2001). This technique can be used to analyze tens of thousands of genes simultaneously. Simple modification of the probes on the microarray chip allows for the probing of different targets. This ability to analyze for a large variety of targets is becoming vital for the detection of the increasing numbers of bio-threat agents and is critical to point-of-care diagnostics.

Traditional microarrays use a labeled target system in which each target DNA/RNA is directly or indirectly (e.g., sandwich hybridization) labeled with a dye. The quantity of each target is monitored by the intensity of each labeling dye. In this process, detection sensitivity depends greatly on the dye performance and the target labeling increases the time commitment and cost of the procedure. By employing cationic conjugated polymers (CCPs), it is possible to both amplify the signal intensity of the existing dyes (**Figure 1A**) and perhaps even eliminate the need for target labeling (**Figure 1B**, **C**).

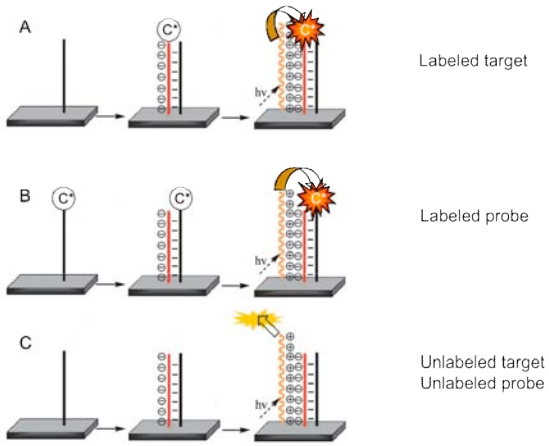

Figure 1. Surface bound detection formats (arrays) under investigation for CCP integration.

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Incorporation of CCPs into microarray platforms can help improve the overall system. For example, current problems with microarrays include poor reproducibility from one test to the next and low signal-to-noise ratios. Our methodology is based on the light-harvesting properties of conjugated polymers. These polymers electrostatically bind to anionic DNA targets, and with their large absorption cross-sections, are able to harvest photons and direct them to the signaling dye (in labeled systems) via FRET. Thus, light output and signal intensity are increased, which, for a point-of-care diagnostic system, should translate to either an increase in sensitivity, or a decrease in the complexity of the detection system.

Homogeneous CCP assays were first developed for the detection of nucleic acids (Gaylord *et al.*, 2002, Gaylord *et al.*, 2003). The CCP materials in these homogeneous assays were shown to amplify fluorescent dye signals via FRET in the event of nucleic acid recognition. This idea was extended to surface-based (heterogeneous) detection platforms, and the first generation of solid-support detection platforms were single spots of PNA, hand-printed and built up step-by-step from an ordinary glass microscope slide (Liu and Bazan, 2005). With these heterogeneous assays, an order of magnitude enhancement was shown in Cy5 emission with complementary labeled DNA (due to FRET), along with a signal-to-noise ratio of five for polymer emission using unlabeled targets. However, slide-to-slide variability was high, primarily because the test spots were hand printed. Initial efforts were further geared toward increasing reproducibility while maintaining the benefits of CCP signal amplification. These considerations form the basis for the study of optically amplified DNA-chips to be developed jointly among Edgewood Chemical Biological Center (ECBC), Sirigen, and UCSB for the rapid detection of pathogenic threat agents.

2. RESULTS AND DISCUSSION

The general array formats under experimental consideration are presented in **Figure 1**. Format A in this series represents a conventional array of DNA probes. This method requires labeling of the target nucleic acid prior to capture and detection steps. Formats B and C represent non-standard PNA probe arrays where detection can be performed without the need for target labeling. This consideration is of critical significance in settings where point-of-care measurements are required. Formats A and B involve energy transfer, or FRET, from the CCP to fluorescent labels on the target and probe molecules, respectively. In each of these cases, CCP is applied to the microarray. The following sections describe the materials used (section 2.1), followed by the integration of these materials into existing commercial DNA arrays and array scanners (section 2.2). Optimization of CCP-enhanced DNA microarrays led to the design of bio-threat probes (section 2.3) and allowed for a transition into custom PNA arrays (section 2.4).

2.1 Cationic Conjugated Polymers

Two different polymers (PFBT and PFP-2F, shown in **Figure 2**) have been synthesized and characterized. Both structures are based on a conjugated backbone with pendant ammonium groups to impart water solubility and an overall cationic charge.

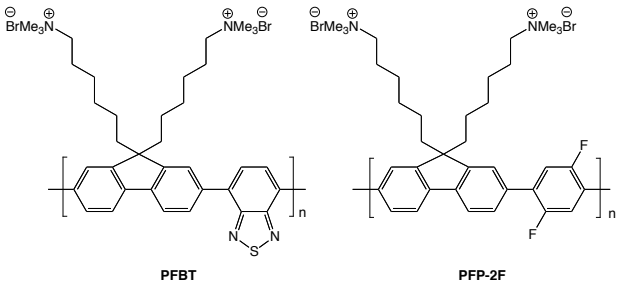

Figure 2. Structures of the two CCPs synthesized for arrays.

The polymer PFBT has a backbone based on a copolymer of fluorene and benzothiadiazole (Liu and Bazan, 2005). The inclusion of the benzothiadiazole units serves to shift the energy levels such that polymer emission is green in color and can act as a FRET donor for dyes with longer excitation wavelengths, such as Cy5. The polymer PFP-2F is based on a fluorene-phenylene backbone, and emits blue in color. Functionalization of the phenyl ring with fluorine allows for better energetic overlap between PFP-2F and dyes such as fluorescein and Texas Red, leading to more efficient energy transfer (Liu and Bazan, 2006). Together, these two polymers can act as FRET donors to a host of dyes spanning nearly the entire visible range and potentially allow for multi-color emission with only two excitation wavelengths.

Additionally, such polymers possess light-collecting properties. These polymers electrostatically bind to the anionic DNA targets, and with their large absorption cross-sections, are able to harvest photons and direct them to the signaling dye (in labeled systems) via FRET. This increases light output and thus signal intensity. To date, a 75-fold amplification (as compared with direct excitation) in fluorescent signaling has been achieved in solution as depicted in **Figure 3**.

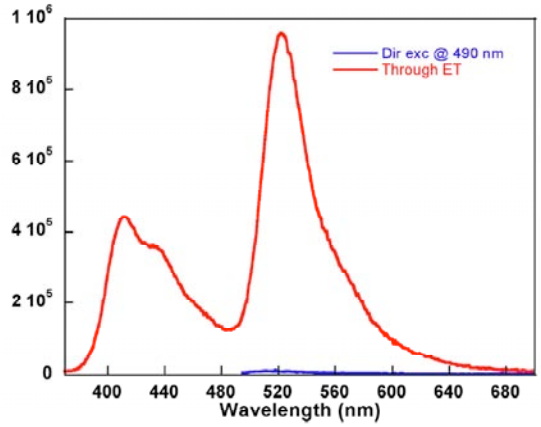

Figure 3. Optical enhancement of fluorescein (red line) via CCP FRET excitation relative to the unamplified fluorescein signal (blue line).

2.2 DNA Microarrays

Since DNA probes represent the most common microarray detection format that can adapt a wide variety of modifications, it serves well as the starting point for validating the compatibility of the CCP materials in such assays. MWG human starter arrays, human genome Agilent arrays, and internally printed quality control slides from Invitrogen were tested for this purpose. These array sources cover a wide range of different commercial suppliers and standard slide chemistries. In addition, several commercial scanners were evaluated. These systems were screened based on scanner sensitivity, image quality, fluorescent cross-talk between polymers and dyes, general market acceptance and, most importantly, the basic compatibility with the available CCP structures. Detailed testing yielded the best results using the Agilent genome slides in a Perkin Elmer fluorescent array scanner. A successful representation of the Agilent/CCP results is shown in **Figure 4A**.

After hybridization and washing, the arrays were treated with a CCP solution and followed by a brief rinse. Signals were detected by array scanner and the results showed a negligible background (from the surface), which allows for straight forward data interpretation without the need for complicated background corrections. The Agilent surfaces provided the lowest background of the DNA arrays tested to date. Furthermore, the data in **Figure 4B** represents the excellent correlation between standard Cy5 signals on the array compared to those generated by CCP amplification. It should be noted that the actual intensity numbers listed are not directly comparable. There is a significant difference in laser power between the two measurements to account for the weak signals generated by conventional microarray dyes (Cy5 in particular). Estimations based on rough internal instrument calibrations indicate that the FRET signals (via CCP excitation) are amplified by at least a factor of three in this initial demonstration. This indicates that stronger signals can be generated with a weaker light source (*i.e.* less power). More significantly, the signal-to-background values have not been adversely altered by the introduction of the CCP amplification. The system does not sacrifice selectivity at the expense of sensitivity, which is often a key trade-off. This is a critical consideration in the design of sensitive yet accurate detection platforms.

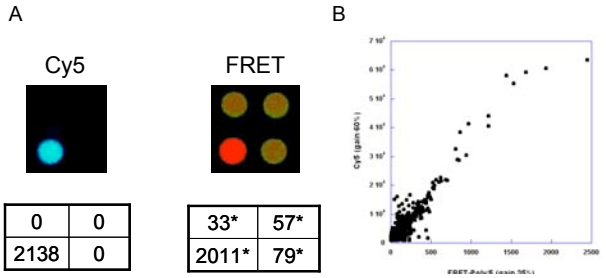

Figure 4. (a) Selected spots from an Agilent 22K spot human genome array. Four spots are shown in each image; the lower left-hand spot shows specific hybridization to Cy5 labeled target and the other three spots are non-specific for the target. Cy5 scan: λ_{ex} = 649 nm, λ_{em} = 670 nm; FRET scan: λ_{ex} = 488 nm, λ_{em} = 670 nm. (b) Correlation of Cy5 intensities arising from direct excitation vs. FRET. *A 20% cross talk correction performed.

Another positive attribute of the DNA arrays depicted in **Figure 4** is the extremely uniform binding of the CCP on each of the spots. The uniformity allows a reliable correction to be made for the small spectral overlap (<20%) observed between the polymer and dye emission peaks (**Figure 4**). This overlap is commonly referred to as "cross-talk", and may be accounted for by using a standard and simple correction employed in most FRET and solution based multiplex assays. Since the ultimate goal of the project is to create highly robust multiplexed tests, the minimization of variability across the array surface is imperative. In this example, there are over 20,000 spots on each of the tested microarrays and any statistically significant variation among spots would have made data interpretation exceedingly difficult.

Similar CCP array results were obtained on MWG human starter arrays (**Figure 5**). The arrays are printed using different techniques and with different surface chemistries indicating the versatility of the general detection method. Again, after applying a straightforward cross-talk correction, optically enhanced signals are obtained without a loss in signal to noise. The data, like that in the Agilent system, correlate linearly with the amount of Cy5 labeled target bound to the DNA probes.

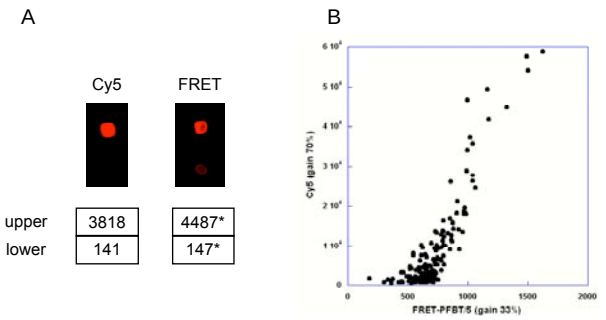

Figure 5. (a) Selected spots from an MWG human starter arrays. Each image represent one spot with specific target hybridization of Cy5 labeled target and one non-specific spot. Cy5 scan: λ_{ex} = 649 nm, λ_{em} = 670 nm; FRET scan: λ_{ex} = 488 nm, λ_{em} = 670 nm. (b) Correlation of Cy5 intensities arising from direct excitation vs. FRET. *20% cross talk correction performed.

Although successful in demonstrating the integration of CCPs within commercial DNA arrays, several steps to address signal cross-talk have been initiated to increase performance and simplify the overall detection process. Because the probes for these commercial arrays are comprised of DNA and are therefore anionic, nonspecific electrostatic binding of CCP to these probes is inevitable. Any overlapping polymer/dye signals on these spots would be considered cross-talk. Two methods have been currently explored for the reduction of cross-talk between the CCP and dye: (a) increase the spectral resolution between the two and/or (b) decrease nonspecific association of CCP to probe. The first idea is demonstrated in **Figure 6**, in which the emission band of the dye (Alexa 750) is red-shifted relative to Cy5 and thus spectrally resolves the emission band of the two reporters (CCP and Alexa 750). Data collected with the Alexa 750 labeled DNA indicate higher levels of optical enhancement relative to the Cy5 labels upon CCP excitation. By labeling the target with Alexa 750 (whose emission maximum is 110 nm red-shifted from Cy5) the cross-talk between the dye and polymer is nearly eliminated (<1%). This in turn leads to true dye signals for probes with non-complementary targets, even with significant binding of CCP to the non-target DNA occurs. Even in cases where the polymer signal saturates the detector, no appreciable FRET signal is observed. Solution and solid state experiments with a blue shifted PFP-2F polymer structure have also been performed. Dyes such as Alexa 555 and Texas Red achieve the same result of reducing cross-talk while retaining the optical amplification afforded by the introduction of the CCP materials (data not shown). Continued studies will be done to map out other possible polymer/dye combinations. The reduced cross-talk effect translates into increased specificity and improved potential for multiplexing several signals.

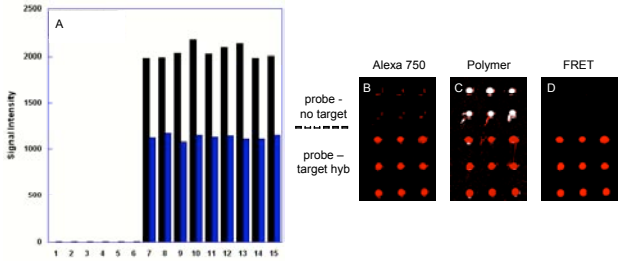

Figure 6. (a) Signal intensity arising from Alexa 750 by direct excitation (blue) and by FRET (black). Bars 1 through 6 indicate probes with nonspecific target and bars 7 through 15 indicate probes with specific targets. Images obtained from array scanner: (b) Alexa 750 scan: λ_{ex} = 633 nm, λ_{em} = 780 nm; (c) polymer scan: λ_{ex} = 488 nm, λ_{em} = 578 nm, white spots indicate signal saturation; (d) FRET scan: λ_{ex} = 488 nm, λ_{em} = 780 nm.

2.3 *Probe Design*

With sufficient data to show CCP compatibility with array technology, the next step was to design a series of probes that were pertinent to the Army's needs, namely, bio-threat agents. Two panels of arrays were designed that have direct applicability to in-field diagnostics: a "Staph" panel and a "Threat" panel. The Staph panel was designed to test strain specificity and includes probes for *Staphylococcus* strains *Staphylococcus epidermis*, SE, *Staphylococcus aureus* enterotoxin A, SEA, and *Staphylococcus aureus* enterotoxin B, SEB. The Threat panel has been designed to test species specificity and includes probes designed for *Bacillus anthracis*, *Bacillus cereus*, *Francisella tularensis*, *Yersinia enterocolitica*, and *Yersinia pestis*.

Probes with high specificity were designed using several readily available software tools. In the case of the development of probes with strain-level specificity (the Staph panel), the published DNA sequences of *Staphylococcus aureus* were aligned using the Meg Align program (DNA STAR, Inc). Sections of sequence containing the highest degrees of conservation within the strains were selected as candidates. These candidate sections were then placed into NCBI nucleotide-nucleotide BLAST to find any closely related organisms (e.g., *Staphylococcus aureus* versus *Staphylococcus epidermidis*). The sequences that were best able to discriminate the target strain from, and least homologous to, those of the closely related strains were selected as probe candidates. From these areas, 15-mers were chosen based on their highest degree of uniqueness after being run through NCBI BLAST search for short, nearly exact matches program. This step aided in determining the degree of cross-hybridization potential with all other published DNA sequences in the world. They were then run through PNA Probe Designer (Applied Biosystems)

to assure that the 15mers met all the criteria to serve as good PNA probes. The species-specific probes were designed in the same fashion as the strain specific probes.

2.4 PNA Microarrays

There has been sufficient progress made on the DNA microarrays to allow us initiate the first set of prototype PNA probe microarrays. These test arrays incorporate the capabilities of detecting both strain-specific and sequence-specific bio-threat agents developed in conjunction with the team at ECBC for this program.

As mentioned previously (section 2.2), the reduction of cross-talk can greatly decrease the complexity of detection. Increasing the spectral resolution between the donor CCP and acceptor dye is one method to simplify analysis. A second route to cross-talk reduction involves decreasing the nonspecific electrostatic binding of CCP to actual probe molecules. The use of charge-neutral PNA probes was introduced in the work describing homogeneous detection assays (Gaylord *et al.*, 2002), and may be a viable method to resolve the cross-talk issue. The basic concept is schematically depicted in **Figure 1B** and **Figure 1C**. The major advantage of this method is the elimination of the need for target labeling prior to capture and detection. The ability to detect unlabeled targets can significantly reduce both the amount of sample preparation and time requirements for the assay, and can lessen the complexity of the detection process. The possibilities for unlabeled target detection include CCP FRET to a labeled PNA probe (**Figure 1B**) and the direct detection of CCP emission (**Figure 1C**), bypassing labeling completely.

Another potential advantage of using the unlabeled PNA system lies in the properties of the actual reporting element. The photo-stability of a standard microarray dye (Cy5) was compared with one of the CCP structures currently under development. The two fluorescent materials were scanned repeatedly over time (**Figure 7**). Significant degradation (>60%) was observed for the dye after a single scan while the CCP retained nearly 85% of its original intensity after nine scans at the maximum laser setting in a commercial scanner. For utilization in point of care or field deployable testing the fluorescent materials must be extremely robust. Conventional dyes like Cy5 are notoriously susceptible to degradation. These results clearly demonstrate the advantage of the proposed unlabeled CCP sensor and the shortcomings of the conventional fluorescent materials.

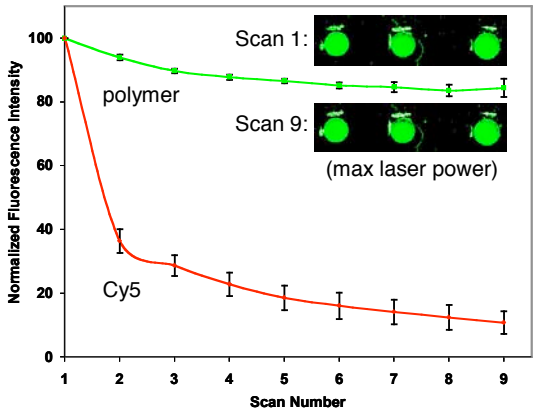

Figure 7. Photo-stability comparison of a conventional microarray dye (Cy5, red line) relative to signals generated by CCP association to unlabeled DNA targets (green line).

An increase in length of the target is naturally accompanied by an increase in charge. Because the assay is based on electrostatic attraction between the CCP and target, higher charge density might attract more CCP to generate higher fluorescent signals in an unlabeled format. This has been demonstrated in **Figure 8**. Longer DNA targets (unlabeled PCR amplicons) tend to bind more polymer, resulting in higher fluorescent signals in the (unlabeled) PNA/CCP assay. This offers a significant advantage in the detection of large nucleic acid targets such as fragmented genomic DNA.

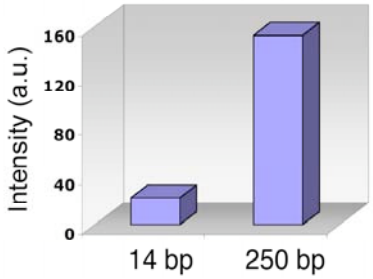

Figure 8. CCP signal intensity as a function of DNA target length in a label-free assay.

A preliminary investigation into the reusability of a hand-printed PNA array slides was recently conducted. Unlike DNA or RNA materials used in conventional microarrays, PNA is a synthetic material and therefore is much less susceptible to degradation by nucleases or proteases. This is a main criterion when designing a field deployable system robust enough to withstand harsh conditions for potentially long periods of time. Several denaturing conditions were investigated, including boiling, treatment with low pH solutions, and treatment with urea solutions. Initial results are shown in **Figure 9**, which shows the signal intensities for the second and fifth cycle of hybridization/heat denaturing (boiling)

process. Signal degradation was observed over several cycles but could be compensated by increasing hybridization time (data not shown).

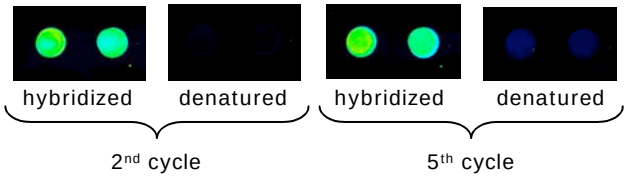

Figure 9. Second and fifth hybridization and denaturing cycles for PNA arrays.

Detection of unlabeled *Staphylococcus* targets by a PNA Staph panel array is shown in **Figure 10**. After optimization of hybridization, washing, and CCP application protocols, good signal-to-noise ratios (greater than forty) between the specific and nonspecific are achieved.

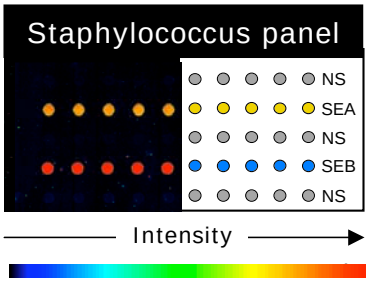

Figure 10. SEA (yellow) and SEB (blue) targets (2×10^{-11} mol) show highly specific signals.

The threshold of detection for these PNA arrays is currently set at 2×10^{-12} mol, as shown in **Figure 11**. At this concentration of target, a 30-minute hybridization results in signal-to-noise ratios greater than five. Indeed, it is possible to detect targets at even lower concentrations; however, this requires increased hybridization times, and results in lower (though still detectable) signal to noise ratios.

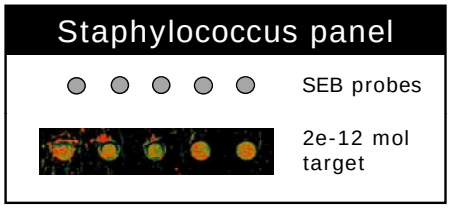

Figure 11. Good signals are shown for target concentrations as low 2×10^{-12} mol with a hybridization time of 30 minutes.

CONCLUSIONS

The application of CCP to commercial DNA arrays has been demonstrated in a commercial off-the-shelf instrument. A 3-fold initial amplification of dye signals via FRET was shown, along with good correlation between standard and FRET-induced signals. The transition to custom PNA arrays has been made, with two bio-threat panels designed and one printed (Staph), and these arrays are currently undergoing testing. Unlabeled target systems have been demonstrated with PNA arrays, with a lower limit of detection set at 2×10^{-12} mol for a 30 minute hybridization time.

The next steps involve the continued integration of probe sequences along with the optimization of sample preparation steps required to perform the standard assays from an actual sample, including target extraction, purification and amplification. Work will continue on furthering the capture and detection steps with a goal to maximize the effectiveness of the CCP detection method coupled with specifically tailored sample preparation steps to minimize the demand on a measuring device *(i.e.* low power, disposable, cost-effective, portable).

The optimization of performance in materials will continue with the aim of reducing nonspecific interactions between the CCP and PNA such that hydrophobic interactions between the two are minimized while electrostatic interactions between the CCP and target DNA are maximized. These adjustments to material development should translate to higher signals or a simplified measuring device.

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